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The Ets family transcription factors are downstream targets of most activated signal transduction steps identified in tumor cells. Interfering with Ets-dependent transcription by expression of the Ets2 DNA binding domain (Ets2DBD) can inhibit or reverse Ras-mediated cellular transformation. Thus, altering Ets activity may be an effective way to reverse the effects of aberrant signal transduction pathways in breast cancer. As a model system, we analyzed the effects of stably expressing a variety of Ets2 constructs in Ras-transformed NIH3T3 (DT) cells. Similar to the Ets2DBD, expression of just the Ets2 transactivation domains strongly inhibited DT cell anchorage-independent growth, but unlike the Ets2DBD, did not reverse the transformed cell morphology. Unexpectedly, high expression of full-length Ets2, a transcriptional activator, broadly reversed the transformed properties of DT cells, including anchorage independent growth, transformed morphology, and tumorigenicity, without impairing attached cell growth. Furthermore, increasing full-length Ets2 transcriptional activity by fusing it to the VP16 transactivation domain enhanced its ability to reverse DT cell transformation. These findings form a strong basis for our current studies examining the effects of either inhibiting or stimulating Ets-dependent transcription on the transformed state of human breast tumor cell lines.

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Gabriela Flores 1/27/99

PI - Signature Date

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Introduction and Background

Breast cancer is one of the major causes of death for women. Human cancers occur as the result of multiple genetic alterations eventually leading to a tumorigenic phenotype. One frequently found alteration in tumors is the constitutive activation of the Ras signaling pathway, often through mutation of Ras. The presence of oncogenic Ras causes widespread changes in cell growth and behavior. The targets of oncogenic Ras signaling which cause the fully transformed phenotype remain unclear, but some of the most downstream targets that have been characterized are transcription factors, whose altered activities can mediate widespread changes in gene expression.

Ets proteins as targets of the Ras signaling pathway. The Ets family of transcription factors share a conserved DNA binding domain. Several Ets proteins are targets for the Ras signaling pathway. The transcriptional activity of the Elk family of Ets ternary complex factors are regulated by the Neu/Ras/Raf/MEK/MAP kinase pathway (1). We found that Ets1 and Ets2 are transcriptionally activated by Neu/ErbB-2 and Ras, requiring specific phosphorylation of a conserved Ets threonine residue (2-4). We found that transcription activation activity of other Ets family proteins such as Fli-1, Elf-1, PEA3, and Pu.1 is not increased by Ras (3). Some Ets factors, such as ERF and Net, are negative regulators of transcription, and their negative regulation can be relieved by stimulation of the Ras signal pathway (5,6).

Altered Ets family transcriptional activity is essential for cellular transformation
The members of the Ets transcription factor family are essential targets for cellular transformation of rodent fibroblasts. There is a strong correlation between the ability of non-nuclear oncogenes to transform cells and to activate reporter genes containing Ets and AP-1 binding sites. Virtually all of the dominant negative mutants that block the Ras-mediated transformation also block transactivation of Ets and AP-1 containing reporter genes. The most direct evidence that the transcriptional activation of Ets and AP-1 families is important for cellular transformation comes from studies with dominant negative mutants of Ets or AP-1. In rodent fibroblasts we and others demonstrated that these mutants (4,7,8) are able to block or even reverse Ras-mediated cellular transformation of rodent fibroblasts without disturbing normal growth of these cells. Because most Ets transcription factors have similar binding specificities, dominant negative mutants which consist of the Ets DNA binding domain, may act as broad inhibitors of the Ets family. These results suggest that the Ets family members are essential for mediating the effects of non-nuclear, transforming oncogenes.

Mutant Ets proteins can block or reverse the transformed phenotype of tumor cells
The expression of dominant mutants for AP-1 has been reported to inhibit the tumorigenicity of breast cancer and epidermal tumor cell lines (9). Importantly, overexpression of Ets1 can reverse the transformed phenotype and reduce the tumorigenicity of a human colon cancer cell line (10) which did not previously express Ets1. However, the growth rate of the cells was not inhibited by this Ets1 overexpression. Recent studies show that the Ets2 transcription factor plays an important role in the regulation of anchorage independent growth and invasiveness of BT20 breast cancer cells (11). These findings show that perturbing Ets activity can revert the transformed phenotype of spontaneous multi-hit human epithelial-derived cells. Taken together these results encouraged me to investigate whether expression of Ets mutants can reverse the transformed phenotype of human epithelial-derived breast tumor cell lines.

Ets activation is implicated in metastasis. The expression of many genes associated with metastasis is increased by activated Ras and other oncogenes. Several of the promoters of these genes contain Ets and AP-1 sites which mediate their Ras-induced activation (12). In particular, extracellular metalloproteinases such as collagenase and stromelysin, which are involved in invasiveness (13,14) are transcriptionally activated by Ets proteins (12). Our lab has shown that the metalloproteinase urokinase-type plasminogen activator (uPA) promoter contains an Ets binding site that mediates strong activation by Ets2 and Ras (3). We also demonstrated that the

enhancer for keratin 18, a diagnostic marker found in 90% of invasive breast carcinomas, also contains a Ras and Ets2-responsive element (15). Thus, I proposed to test the effects of inhibiting Ets activity on invasiveness both *in vitro* and *in vivo*.

OBJECTIVES/HYPOTHESES

The overall goal of this proposal is to determine whether altering Ets activity, by expression of mutant forms of Ets2, will specifically reverse the transformed phenotype of breast cancer cells. This analysis will determine whether the Ets family of transcription factors is a novel downstream target for therapeutic intervention in breast tumors.
The hypotheses are 1) that Ets transcription factors are downstream targets for multiple signal transduction pathways in the transformation of breast epithelial cells and 2) that interfering by expression of Ets mutants reverses the transformed phenotype and/or metastatic potential of breast cancer cells.

Progress: The majority of objective 1 has been completed, and construction of the stable cell lines for objective 2 is underway.

Objective 1: Comparison of the effect of Ets2 mutants in reversing transformation in rodent fibroblasts and human breast epithelial cells.

Milestone 1: Generation of stable Ets-expressing DT cell lines. To characterize the role of Ets proteins in Ras-mediated cellular transformation, a variety of Ets2 expression constructs were generated, including expression constructs for full-length Ets2, the Ets2 transactivation domains (Ets2TAD), and the Ets2 DNA binding domain (Ets2DBD). A schematic diagram of these constructs is shown in Figure 1A. The ability of these Ets2 expression constructs to reverse Ras-mediated transformation was analyzed in stable clones generated in the DT cell line, a v-Ki-Ras transformed derivative of mouse NIH3T3 fibroblasts (16). To improve the frequency of individual stable drug-resistant colonies expressing high levels of the Ets2 constructs, I used the pCIN4 vector (17). The high frequency of cell lines expressing the introduced Ets2 constructs allowed us to directly analyze the phenotype of stable clones, without preselecting for adherent revertants as was previously described for the Ets2 DNA binding domain (8). For the analysis described below, multiple individual stable DT cell clones generated with each Ets2 expression construct or control plasmid were randomly picked, expanded, and assayed for alterations in the transformed phenotype and for expression of the introduced gene product. To exclude clonal variations I established and analyzed at least 6 cell lines for each construct (about 100 total). This was more time consuming than expected, but it helped me identifying the Ets2 constructs with the highest reversion potential and forms a solid basis for objective number 2.

Milestone 2: Expression levels of Ets2 constructs in reverted cell lines.
Representative analysis of three randomly selected full-length Ets2 cell lines, six Ets2DBD cell lines and 3 VP16Ets2 lines were assayed for Ets2DBD protein expression (Fig. 2). All of the G418-resistant cell lines tested expressed protein detectable by western blotting.

Milestone 3: Expression of full-length Ets2, Ets2TAD, or Ets2DBD inhibits anchorage-independent growth of DT cells. Soft agar assays were used to analyze the capacity for anchorage-independent growth of individual DT cell clones transfected with either empty pCIN vector or the pCIN-Ets2 expression constructs. The results of this analysis are shown in Fig. 1B/C. None of the cell lines derived with the empty pCIN expression vector displayed significantly reduced growth in soft agar relative to the parental DT cells, but surprisingly, 7/11 of the cell lines derived with full length Ets2 showed a greater than a 4-fold reduction in soft agar growth. The majority of clones derived with the Ets2TAD, which lacks the Ets2 DNA binding domain, also showed significantly reduced anchorage-independent growth.

Finally, similar to a previous report (8), several Ets2DBD cell lines also showed significant reduction in anchorage-independent growth (Fig. 1C). To determine that reduced soft agar growth reflected a specific loss of anchorage-independent growth and not impaired attached cell growth or errors in cell counting, cells from the same dilution used for the soft agar assay were plated in parallel onto normal tissue culture dishes. The results show a clear reduction in soft agar growth but not in attached growth. In addition I found that the growth rates of cell lines expressing full-length Ets2 or the Ets2 TAD were similar to that of control lines or the parental DT cells during exponential growth. However, consistent with a more reverted phenotype, the cell lines with Ets2 expression constructs exhibited a reduced saturation density. Together, these data show that expression of either full-length Ets2 or the Ets2 transactivation domains can strongly and specifically inhibit anchorage-independent growth of DT cells. Comparison of the Ets2DBD expression levels and the reduction of soft agar growth in these cell lines shows that there is indeed a correlation between Ets2DBD expression and apparent reversion in these cell lines. Because DT cells already express full-length Ets2, reversion of the transformed phenotype by the introduced full-length Ets2 expression construct is likely due to the elevated levels of Ets2 present in the reverted cell lines. To measure the amount of introduced full-length Ets2 mRNA relative to the endogenous Ets2 levels, RNase protection assays were performed with a probe that could distinguish between the endogenous Ets2 and the FN-tagged introduced Ets2. To determine how much overexpression was required for reversion, I tested a full-length Ets2 cell line that was only moderately reverted (E2full#2), and two others that showed strongly reduced growth in soft agar (E2full#5 and #6). I estimate that approximately 10-fold overexpression of Ets2 is required for strong reversion activity in DT cells. VP16Ets2full protein levels were also analyzed in several clones, and there was about 4-fold more VP16E2full#7 than Ets2full#5 (not shown). Because the reverted VP16Ets2full clones all had higher stable levels of protein expression than the Ets2 full clones, I can not exclude the possibility that the enhanced apparent reversion activity of the strongly transactivating VP16Ets2 constructs was due to dosage effects.

Morphological reversion of DT cells by expression of full-length Ets2 or Ets2DBD, but not Ets2TAD. The cell morphology of stable cell lines found to exhibit significantly reduced anchorage-independent growth was examined using phase-contrast microscopy of live cells. Normal NIH3T3 cells exhibit a flat, non-refractile morphology, whereas the Ras transformed DT cells containing empty pCIN expression vector are spindly and refractile (Fig. 3), as are the parental DT cells. However, I found that all of the full-length Ets2 or the Ets2DBD DT cell lines which exhibited significantly reduced soft agar growth were flat and non-refractile. Interestingly, this apparent morphological reversion was not observed in any of the cell lines expressing the Ets2TAD, despite the fact that they showed reduced growth in soft agar. In addition, I analyzed the cytoskeletal organization of the various cell lines after staining with rhodamine-conjugated phalloidin. As previously observed, NIH3T3 cells contained well organized actin stress fibers, which are not present in Ras transformed DT cells (Fig.3). Similar to the changes in overall cell morphology, the actin stress fibers reappeared in DT cell lines expressing full length Ets2 or the Ets2DBD, but not in cells expressing the Ets2TAD. Thus, unlike expression of full-length Ets2 or the Ets2DBD, expression of the Ets2 TAD is unable to cause reversion of cell morphology or actin filament reorganization in DT cells, indicating that there are differences in the targets of these Ets2 constructs.

The Ets2 (thr72) residue is not required for reversion activity. We previously showed that phosphorylation of the Ets2 (T72) residue is essential for the Ras pathway-mediated increase in Ets2 transactivation activity, and that an Ets2(A72) mutant retained basal transcriptional activity, but lost Ras-responsiveness (4,18). To test the connection between Ras signaling to Ets2 and the ability of expressed Ets2 to revert transformation, I determined whether expression of Ets2TAD(A72) or full-length Ets2(A72) could reverse the anchorage-independent growth or transformed morphology of DT cells. While introduction of the (A72) mutation caused a modest reduction in apparent reversion activity, both the full-length Ets2(A72) and the Ets2TAD(A72) constructs still clearly caused significant decreases in anchorage-independent growth indicating that

phosphorylation of Ets2 (thr72) was not essential for their reversion activity. Analysis of the effects of full-length Ets2(A72) or the Ets2TAD(A72) on cell morphology and actin stress fibers revealed that these (A72) constructs had the same effect as their wild-type counterparts: the full-length Ets2(A72) cell lines appeared reverted, and the Ets2TAD(A72) cell lines did not.

Ets2 constructs act downstream of Ras expression or MAP kinase activation. To determine that the Ets2 constructs were not reverting the transformed phenotype of DT cells by simply extinguishing the expression of oncogenic Ras, I confirmed that the high level of Ras normally seen in DT cells was still present in the reverted clones by western blot using the pan-Ras antibody (not shown). The results show that the reverted cell lines expressing either full-length Ets2 or the Ets2TAD still expressed high levels of Ras similar to that of DT cells. Therefore, the effects on the transformed phenotype by the Ets2 constructs were not due to reduced Ras expression. The expressed Ets2 proteins are likely to act well downstream of Ras, and I postulated that they would also act downstream of the MAP kinases ERK1 and ERK2. These ERKs are targets of Ras/Raf/MEK signaling, but are still upstream regulators of Ets2 transcriptional activity (19). I therefore analyzed MAP kinase activity in several cell lines, using an immune complex assay. There was not a significant difference between the basal levels of ERK activity found in the parental DT cells and in representative Ets2 construct-reverted cell lines, indicating that the Ets2 constructs were not altering ERK activity in the reverted cell lines (not shown).

Determine whether altered Ets activity can revert the transformed phenotype of MCF-10Tneo cells. An unexpectedly large amount of effort was required to establish and analyze multiple independent cell lines of the DT fibroblasts expressing each of the various Ets2 constructs. This effort was well spent, as it led to novel insights into the role of Ets factors in cellular transformation (29). I proposed to subsequently establish similar cell lines in the experimentally Ras transformed breast tumor cell line MCF-10Tneo (20) While the connection of this experimentally generated cell line to actual breast cancer is not straightforward, it offered a model cell line with a well understood defect in signal transduction. However, in light the recent findings that inhibiting Ets activity can reverse aspects of the transformed phenotype of several types of human tumor cells not directly associated with oncogenic Ras (11,21), I have decided it would be more productive to move directly to generating and analyzing stable Ets2 construct-expressing human breast tumor cell lines (Objective2) .

Objective 2: Reversion of the transformed phenotype of breast cancer cell lines.
Milestone 5: Establishing breast cancer cell lines stably expressing Ets mutants. The extensive analysis in objective 1 helped me to identify the Ets2 constructs that are effective in reversing the transformed phenotype of Ras transformed cells. As an initial step I analyzed a panel of breast cancer cell lines for their ability to grow in soft agar and optimized their growth conditions. I chose the highly invasive breast cancer cell lines MD-MBA-435 to start the analysis. Currently, I am establishing MD-MBA-435 cell lines stably expressing the activating constructs full-length Ets2 and VP16Ets2 and the dominant inhibitory construct Ets2DBD and the empty vector pCIN. In addition, I am establishing similar cell lines in the untransformed but immortal MCF-10A breast cells, to determine which effects I observe with the dominant Ets constructs (*e.g.* apoptosis) are tumor cell-specific.

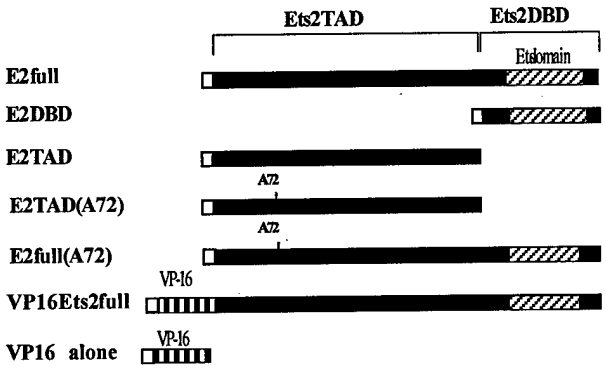


Figure 1A: Schematic diagram of Ets2 constructs

Cell Line	Tumor Volume (mm^3) day 4	day 6	day 8	day 10
pCIN5	19±17	263± 159	1,331 ±649	2,816 ±1,272
full#5	4±7	51± 39	390 ±246	1,223 ±661
full#6	8±16	103 ± 62	599 ±379	1,467 ±949
DBD#10	16±11	147± 98	479 ±228	1,736 ±1,138
TAD#53	8±16	49± 51	357 ±277	1,618 ±1,004
VP16full#1	22±13	76± 62	630 ±516	1,553 ±973
VP16full#7	7±8	93± 87	255 ±245	1,197 ±1,056

Table 1: Tumor formation in nude mice

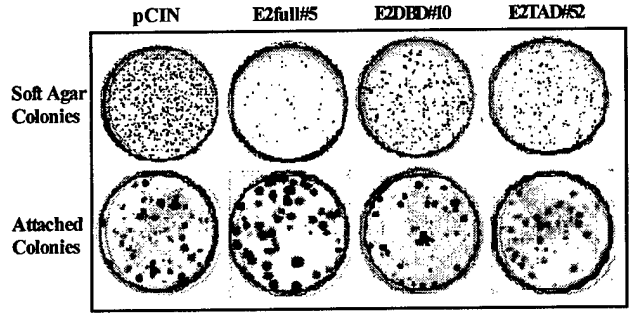
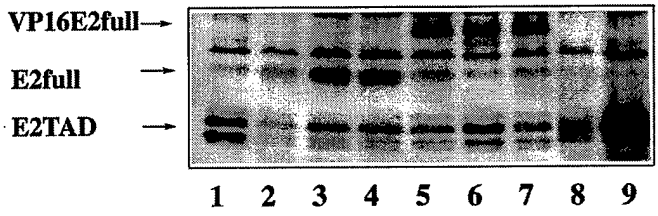


Figure1B: Soft agar and attached growth of individual stable DT clones

DT cells transfected with:	Number of stable lines with indicated soft agar colony formation (relative to DT) >50%	25-50%	10-25%	<10%
pCIN	5	0	0	0
Ets2full	2	1	4	4
Ets2DBD	6	1	5	3
EtsTAD	1	0	4	2
E2TADA72	1	2	2	1
E2FullA72	0	2	2	1
VP16-E2full	0	0	0	10
VP16 only	6	1	0	2

Figure 1C: Percent soft agar colony formation relative to DT cells



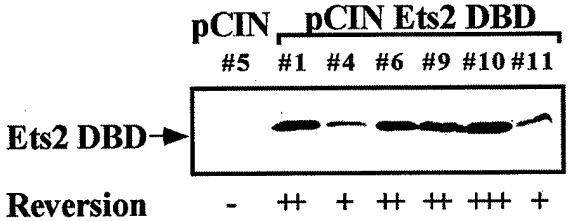
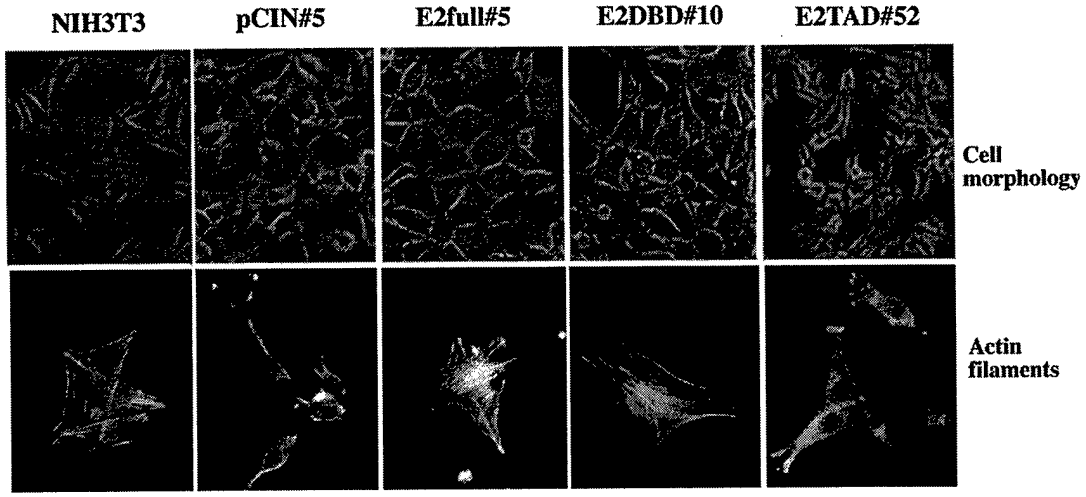


Figure 2: Expression levels of Ets2 construcs in stable DT cell lines

Figure 3



Objective 3: *In vivo* analysis of the reversion of tumorigenicity and metastasis induced by Ets2 mutants.

Ets2 expression reduces tumorigenicity of DT cells in nude mice. As background for objective 3 we determined whether the Ets2-mediated morphological reversion or increased anchorage-dependence of DT cell lines seen *in vitro* reflects a reduced ability to form tumors. With these analysis I gained experience in *in vivo* studies that will be of advantage for analyzing the *in vivo* effects of the breast cancer cell lines. In tumorigenicity assays in nude mice we saw that the rapid tumor growth of the pCIN#5 cell line was not significantly different than another empty vector DT cell line pCIN#6 (n=8), or to the parental DT line (n=4), (data not shown). However, the tumor volumes were significantly smaller than pCIN#5 for both full-length Ets2 cell lines and both VP16-Ets2 cell lines on days 6, 8, and 10 (Table 1). Reductions in tumor growth were also seen in the Ets2TAD#53 and Ets2DBD#10 cell lines, but the smaller tumor volumes were statistically significant only on days 6 and 8 for the Ets2TAD line and only on day 8 for the Ets2DBD line. Due to the very high initial growth rate of the control cell lines, by day 10 the tumors from pCIN or parental DT cells were quite large, and started to exhibit reduced growth rates. This allowed the delayed tumors derived from the Ets2 construct-containing cell lines to catch up, and by days 12 and 14, none of the tumor volumes were significantly different from those of the pCIN#5 line (data not shown). It is likely that the significant delay of tumor growth represents the effects of the expressed Ets2 constructs, but in the absence of G418 selection, the subsequent *in vivo* selection of cells in which expression from the pCIN vector is reduced. Overall, the ability of full-length Ets2 or VP16-Ets2 expression to cause a substantial delay in tumorigenicity, and the ability of the Ets2TAD to cause a shorter delay in tumorigenicity correlates with their relative ability to cause reversal of the transformed phenotype in cell culture experiments.

Discussion

During the first year of my fellowship I showed that elevated expression of full-length Ets2, an activator of Ets-dependent transcription, can also specifically reverse the transformed phenotype of Ras-transformed NIH3T3 cells. The reverted phenotype in multiple stable Ets2-expressing cell lines included a reduction in anchorage-dependent growth, tumorigenicity, and saturation density. In addition, these Ets2 reverted cell lines exhibited a more normal cell morphology, and reappearance of actin stress fibers which are lost in transformed fibroblasts.

To test a potentially more specific inhibitory construct for Ets-dependent transcription, I also assayed the reversion activity of expressing the portion of Ets2 which does not bind DNA, the Ets2TAD. In contrast to the ability of the Ets2DBD or full-length Ets2 to more completely reverse transformation, I showed that expression of Ets2TAD partially reversed the transformed phenotype of DT cells. Ets2TAD expression strongly inhibited anchorage-independent cell growth, but did not reverse transformed cell morphology. These findings suggest that the functions of Ets proteins in anchorage-independence and transformed morphology are separable, and that Ets factors impact on multiple pathways required in cellular transformation.

Because phosphorylation of the Thr72 residue of Ets2 has been found to be essential for the Ras-mediated increase in Ets2 transactivation activity (4,18), I tested whether a Thr to Ala mutation would disrupt the reversion activity of highly expressed Ets2, both in the context of the Ets2 TAD, and in full-length Ets2. I showed that the Thr72 residue was not essential for reversion activity in either construct.

In a collaborative project, we further demonstrated that a prostate cancer cell line PPC-1 can be reverted to anchorage-dependent growth by either full-length Ets2 or the Ets2 DBD. This indicates that although our initial findings are in an experimentally transformed rodent cell line, that distinct approaches to altering Ets activity may indeed be effective in reversing the transformed phenotype of spontaneous arising epithelial-derived human tumor cells. In these studies we further found that

altered Ets activity led to an increase in apoptosis and reduced migration in these human tumor cells.

Inhibition of Ras transformation in fibroblasts has previously been observed only with Ets constructs that inhibit expression of Ets-dependent transcription, and thus it was somewhat unexpected that expression of full-length Ets2 had strong and broad reversion activity. It was previously hypothesized that the Ets DBD acts as a dominant negative Ets protein which blocks the activation of genes whose persistent activation by Ras signaling is required for maintaining the transformed phenotype (22). To assess whether the transactivation activity of the overexpressed Ets2 contributes to its reversion activity, I synthetically increased Ets2 transactivation activity by N-terminal addition of the VP16 transactivation domain. This VP16-Ets2 construct showed the strongest apparent reversion activity of any of the Ets2 constructs I tested, both in the *in vitro* assays and the tumorigenicity assays. These results further indicated that activation of Ets-dependent transcription can also lead to reversion. A related finding was recently reported in the colon cancer cell line DLD-1, where ectopic expression of full-length Ets1 caused a partial reversion of the transformed phenotype of these cells, and an Ets1 mutant that had lost its transactivation activity also lost its reversion activity (10). This ability of highly expressed Ets1 and Ets2 to reverse cellular transformation is in contrast to the initial characterization of Ets1 and Ets2 as proto-oncogenes, whose overexpression could transform NIH3T3 cells (23,24). However, the rare appearance of transformed cells in these experiments suggested that selection for a secondary event was required for transformation (23,24).
Overall, my studies show that altering Ets transcription factor activity by either inhibiting or activating Ets-dependent transcription can reverse Ras-mediated cellular transformation. Therefore, my results with high level expression of Ets2 constructs should not be interpreted to mean that Ets2 is the critical Ets protein in cellular transformation, or that Ets2 is a bona fide tumor suppressor gene. Instead, I have used the Ets2 constructs as tools to explore the potential functions of Ets proteins in Ras transformation, and have found that Ets proteins appear to have a central and complex role in regulating transformation. These findings support my original hypothesis, and make it all the more compelling to extend this analysis to the role of Ets transcription factors in breast tumor cell lines.

Experimental Methods and Material

Plasmids. The pCIN4 mammalian expression vector used for all of the constructs is a derivative of the pCIN plasmid (17), and was kindly provided by Steve Rees (Glaxo Wellcome). This vector is based on pcDNA3 (Invitrogen) and utilizes the CMV enhancer/promoter to express an inserted coding sequence, which is linked by a viral IRES to the neomycin phosphotransferase (NPT) gene. These constructs all include the FN sequence, a 20 residue N-terminal sequence containing the FLAG epitope tag followed by an SV40 nuclear localization signal . The pCIN-Ets2 constructs (from 5' to 3') contained a consensus translational start, the FLAG and SV40 NLS sequences fused to the Ets2 coding sequences, a splice site, an IRES, the NPT gene, and a poly(A) signal. The VP-16-containing constructs were generated using standard PCR methods to insert VP16 residues 410-479 in-frame to the junction of the FLAG/NLS and the Ets2 coding sequences in FN-Ets2, or into the empty FNpCDNA3 vector. These sequences were then transferred to pCIN4.

Soft agar/attached growth assay: Soft agar assays were essentially performed as described (25). Either 500 or 1000 cells were plated in 60 mm dishes in 1.5 ml media containing 0.33% agar, which was overlaid onto 7 ml solidified media containing 0.5% agar. For the DT 3T3 cells the media used for soft agar assays was DMEM + 10% fetal calf serum, and did not contain G418. The soft agar plates were fed with 0.5 ml media every 5-7 days, and after 14 days, the cells were stained overnight (at 37° and 5% CO_2) with the vital dye p-Iodonitrotetrazolium violet (INT), and then counted. In parallel to each soft agar assay, an attached growth assay was performed, where 100 cells from the same cell dilutions were plated on standard tissue culture dishes and grown for 7 days. The resulting attached colonies were stained with crystal violet and counted.

Actin staining and immunoblots Actin filaments were visualized by growing the cells on glass cover slips and staining the cells with rhodamine conjugated phalloidin (Molecular Probes, Inc.) as recommended by the manufacturer. The FLAG epitope tagged Ets2 proteins and the Ras proteins were detected in immunoblots using the anti-FLAG M5 monoclonal antibody (Kodak), and the pan-ras AB-4 (Calbiochem) respectively. Primary antibody binding was visualized using the Phototope-HRP Western Blot Detection Kit (New England BioLabs).

MAPK and RNase protection assays: The immune complex MAP kinase assays were performed essentially as described (26), using 50 μg of total cell extract protein and the ERK1 C-16-G antibody (Santa Cruz Biotechnology). The precipitated MAPK bound to this antibody and protein G sepharose (Pharmacia) was incubated with buffer containing [$g^{32}P$] ATP and 10 μg of myelin basic protein (Sigma). The entire reaction mix was then subjected to SDS-PAGE, and radio labeled MBP was detected by autoradiography and quantitated using a phosphorimager (Bio-Rad). RNase protection assays were performed as described (27) and a probe for the L32 ribosomal protein L32 mRNA was included as an internal standard as described (28).

Tumorigenicity assay: Stable cell lines grown under G418 selection were trypsinized, washed with PBS, and counted. Then, $5x10^5$ cells in 0.1 ml PBS were subcutaneously injected into both the right and left dorsal flanks of 4 week old nude mice. At 2 day intervals, the tumors were measured externally by caliper, and the average of the tumor length and width was estimated to be the tumor diameter, d. The approximate tumor volume was calculated using the formula $V= (\pi/6)(d)^3$. For each cell line, 6-8 tumors were generated (in both flanks of 3-4 mice) and measured over the indicated time. The average tumor volume in mm^3 was then calculated. The statistical analysis of tumor growth was performed by comparing the individual volumes of the 6-8 tumors derived from each cell line relative to that of tumors from DT cells containing the empty expression vector pCIN#5, using a 2-tailed, unpaired t-Test.

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